Cloning and Characterization of upp, a Gene Encoding Uracil Phosphoribosyltransferase from Lactococcus lactis

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Uracil phosphoribosyltransferase catalyzes the key reaction in the salvage of uracil in many microorganisms. The gene encoding uracil phosphoribosyltransferase (upp) was cloned from Lactococcus lactis subsp. cremoris MG1363 by complementation of an Escherichia coli mutant. The gene was sequenced, and the putative amino acid sequence was deduced. The promoter was mapped by both primer extension and analysis of β-galactosidase expressed from strains carrying fusion between upp promoter fragments and the lacZ gene. The results showed that the upp gene was expressed from its own promoter. After in vitro construction of an internal deletion, a upp mutant was constructed by a double-crossover event. This implicated the utilization of a plasmid with a thermosensitive origin of replication and a new and easy way to screen for double crossover events in both gram-positive and gram-negative bacterial strains. The phenotype of the uracil phosphoribosyltransferase-deficient strain was established. Surprisingly, the upp strain is resistant only to very low concentrations of 5-fluorouracil. Secondary mutants in thymidine phosphorylase and thymidine kinase were isolated by selection for resistance to high concentrations of 5-fluorouracil.

A growing organism has a need for nucleotides in order to synthesize DNA, RNA, and several cofactors. This demand can be fulfilled in two ways: (i) by de novo synthesis of the nucleotides or (ii) by utilization of already existing nucleotides, nucleosides, or nucleobases, arising from degradation of RNA and DNA inside the cell or from uptake of precursors present in the growth medium. Lactococci are able to synthesize pyrimidines de novo since they are able to grow on a defined medium in the absence of pyrimidines (32). Furthermore, it has been shown that lactococci are able to utilize different exogenous pyrimidine sources, including uracil (24). The key step in uracil salvage is the reaction of uracil with 5-phosphoribosyl-α-1-pyrophosphate (PRPP), resulting in the formation of UMP and pyrophosphate. The reaction is catalyzed by uracil phosphoribosyltransferase (UPRTase), encoded by upp. This enzyme seems to be present in most microorganisms (see reference 25 for a review). The cloning and sequence determination of the corresponding genes from Escherichia coli (4), Saccharomyces cerevisiae (20), and Streptococcus salivarius (11) have previously been reported. In this report, we describe the cloning, sequencing, and characterization of the upp gene encoding UPRTase from the gram-positive lactic acid bacterial strain Lactococcus lactis MG1363 (9).

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this work are presented in Table 1.

Media, growth conditions, transformation, preparation of extracts, and enzyme assay. Lactococcal cultures were grown either on M17 glucose broth (37) or on SA medium, a morpholinepropanesulfonic acid (MOPS)-based chemically defined medium containing 1% glucose, seven vitamins, and 19 amino acids (32). E. coli cultures were grown on LB broth or on AB medium containing glucose (0.5%), Casamino Acids (1%), thiamine (1 μg/ml), and biotin (1 μg/ml) (6). For plates, agar was added to 15 g/liter. All incubations were at 30°C in the presence of oxygen. When needed, the following were added to the different media: 5-fluorouracil (FU) at 0.3, 1, 5, 10, and 25 μg/ml, uracil at 20 μg/ml, pyrimidine nucleosides at 40 μg/ml, erythromycin at 1 μg/ml for lactococci and 250 μg/ml for E. coli, ampicillin at 100 μg/ml, and tetracycline at 8 μg/ml. L. lactis was transformed by electroporation (15). E. coli cells were transformed as previously described (34). For assays the cells were grown aerobically to 0.8 units of optical density at 450 nm (OD450) and harvested. The cells were washed and resuspended in 50 mM Tris-HCl (pH 7.8)–1 mM EDTA–1 mM dithiothreitol, resulting in a 100-fold concentration of the cells. Lactococcal cells were lysed in the French pressure cell press at 28,000 lb/in2, whereas E. coli cells were lysed by sonication. Cell debris was removed by centrifugation, and the supernatant was used directly for determination of enzyme activity. UPRTase activity was assayed as described by Rasmussen et al. (30). Thymidine kinase and thymidine phosphorylase activities were assayed as previously described (24). Protein determination was performed as described by Lowry et al. (21). For monitoring β-galactosidase activity, cells were grown in M17 glucose broth without aeration at 30°C and assayed at 30°C as previously described (34).

DNA isolation, manipulation, and electrophoretic analysis. Plasmids and chromosomal DNA were isolated as previously described (34). Plasmid DNA was enzymatically manipulated and analyzed by agarose gel electrophoresis as previously described (34), under the conditions recommended by the manufacturers. Chromosomal DNA was digested with restriction enzymes, separated by agarose gel electrophoresis, and analyzed by Southern hybridization (34). DNA sequences were determined by the dideoxy sequencing method of Sanger et al. (35).

Construction of plasmids. The following constructions were made: pJM300 was digested with XbaI and Smal. The protruding end at the XbaI site was filled in by Klenow polymerase...
TABLE 1. Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli BM604</td>
<td>HfrH thi galE Δ(att-l-bio) deoA103 deoC argA pylR upp udp pylRF30</td>
<td>4</td>
</tr>
<tr>
<td>SØ106</td>
<td>lacZ rpsL thi pylRF30</td>
<td>4</td>
</tr>
<tr>
<td>L. lactis MG1363</td>
<td>Prophage cured and plasmid free wild-type strain</td>
<td>9</td>
</tr>
<tr>
<td>MB112</td>
<td>MG1363 derivative carrying a upp deletion</td>
<td>This work</td>
</tr>
<tr>
<td>MB113</td>
<td>MB112 derivative carrying a pylRF mutation</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUN121</td>
<td>Ap′, cI, Tc′ (under control of λ pK)</td>
<td>26</td>
</tr>
<tr>
<td>pG′ host4</td>
<td>Formerly pVE6004, temperature-sensitive derivative of pGK12</td>
<td>5, 23</td>
</tr>
<tr>
<td>pJM300</td>
<td>upp-complementing plasmid; Ap′, Tc′, upp</td>
<td>This work</td>
</tr>
<tr>
<td>pJM306</td>
<td>Same as pJM300 but in the opposite orientation; upp</td>
<td>This work</td>
</tr>
<tr>
<td>pJM307</td>
<td>XbaI-Smal deletion of pJM300; Ap′, Tc′, upp</td>
<td>This work</td>
</tr>
<tr>
<td>pJM307</td>
<td>Ncol-SalI deletion of pJM370; Ap′, upp</td>
<td>This work</td>
</tr>
<tr>
<td>pJM378</td>
<td>EcoRI-EcoRV deletion of pJM371; Ap′, upp</td>
<td>This work</td>
</tr>
<tr>
<td>pG′ 371</td>
<td>PsrI fusion of pJM371 and pG′ host4; Er′, upp</td>
<td>This work</td>
</tr>
<tr>
<td>pG′ 378</td>
<td>PsrI fusion of pJM378 and pG′ host4; Er′, upp</td>
<td>This work</td>
</tr>
<tr>
<td>pIP55</td>
<td>Internal pylRF fragment from MG1363 cloned on pBR322; Ap′</td>
<td>1</td>
</tr>
<tr>
<td>pAK80</td>
<td>lacLM promoter fusion vector; E. coli-L. lactis shuttle vector; Er′</td>
<td>19</td>
</tr>
</tbody>
</table>

Phenol method previously described (method II in reference 10). RNA from L. lactis was extracted by the method developed for Bacillus thuringiensis (12). Primer extensions on 1 µg of total RNA were performed as described previously (4).

Construction of an upp deletion on the lactococcal chromosome. pG′ 378 was transformed to L. lactis MG1363. Transformants were selected and purified on 1 µg of erythromycin per ml at 28°C. To select for integration on the chromosome, the plasmid-carrying cells were streaked on a M17 plate supplied with glucose and erythromycin and incubated at 35°C overnight. Colonies were restreaked on erythromycin plates and incubated overnight at 35°C. Colonies were streaked on erythromycin-containing plates and incubated at 28°C overnight in order to facilitate excision of the plasmid by recombination. M17 medium supplied with glucose and erythromycin was inoculated with erythromycin-resistant colonies and incubated at 28°C overnight. Plasmid DNA was extracted and analyzed by EcoRI digestion and agarose gel electrophoresis. Strains containing plasmids with an EcoRI site in the lactococcal DNA, indicating that the wild-type upp gene has been excised, leaving a deletion on the chromosome, were cured for plasmid by streaking on a M17 glucose plate and incubated at 35°C overnight. Colonies were restreaked on M17 glucose plates, incubated overnight at 35°C, and screened for erythromycin sensitivity. The strain was designated MB112.

Uracil excretion. Strains to be tested were grown in SA glucose minimal medium overnight. The bacteria were removed by centrifugation, the pH was adjusted to 7.0, and the bacteria were subsequently sterilized by filtration through a 0.22-µm-pore-size Millipore filter. The sterile medium was placed in two tubes with 2 ml in each and supplied with ampicillin to 100 µg/ml. One tube was inoculated with 10⁶ cells of the indicator strain BM604 harboring pJM370. The second tube was kept as a control without inoculation.

The assay was standardized in the following way. Two series of tubes were set up, containing 2 ml of fresh medium supplied with ampicillin at 100 µg/ml and increasing amounts of uracil (0 to 4 µg/ml), and were inoculated with BM604 harboring pJM370. All samples were incubated at 37°C overnight. Growth yield was established by measuring OD₄₅₀. The cell densities in the cultures with known amounts of uracil were used to quantify the uracil excretion of the different strains. The presence of 1 µg of uracil per ml facilitates the growth of the E. coli strain to a density of 0.17 OD₄₅₀ unit. This value was also obtained by using medium inoculated with the wild-type lactococcal strain MG1363, grown overnight, and sterilized as described above. The results are the mean values of 16 independent experiments, and the standard deviation were calculated by assuming a normal distribution.

Incorporation of uracil into nucleic acids. Cells were grown in SA glucose minimal medium, and at an OD₄₅₀ of 0.5, 50 µM [2-14C]uracil (5 mCi/mmol) was added. After 0.5, 2, 5, 10, 20, and 30 min, 0.2-ml samples were withdrawn, precipitated with 5% trichloroacetic acid, and filtered as previously described (31).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL data library and assigned accession number X73329.

RESULTS

Cloning of the upp gene. The gene was cloned by gene complementation in E. coli. E. coli BM604 has a pyrimidine requirement, since it is mutated in the pylF gene, and is furthermore unable to utilize uracil because of a upp mutation. This strain is viable, since the pyrimidine requirement can be

Treatment, and the resulting linear molecule was circularized with T4 DNA ligase and transformed into BM604. The obtained plasmid, designated pJM370, was digested with SalI and Ncol, the protruding ends were filled in with Klenow polymerase, the resulting molecule was ligated and transformed into BM604, resulting in pJM371. This plasmid contains the upp gene on a 1.9-kb Ncol-XbaI fragment. This plasmid was digested with EcoRI and EcoRV, the protruding ends were filled in, and the resulting molecule was ligated and transformed into BM604. This plasmid, designated pJM378, thus carries an internal deletion of most of the upp open reading frame. pG′ 371 and pG′ 378 were obtained in the following way. pJM371 and pJM378 were digested with Psrl and ligated with Psrl-digested pG′ host4 (5). The ligation mixtures were used to transform BM604, and the recombinant plasmids were selected directly by plating on LB agar plates containing 250 µg of erythromycin per ml and incubating overnight at 37°C.

In the analysis of transcription initiation the lacLM promoter fusion, vector pAK80, constructed by Eric Johansen, Christian Hansen Laboratory, was used (17, 19). The plasmids fusing the upp promoter to lacLM were constructed in the following way. The HindIII-PvuII fragment of pJM370 was ligated into HindIII-Smal-digested pAK80, thus obtaining pJM333. The cohesive ends of the Ncol-PvuII and NarI-PvuII fragments from pJM370 were filled in with the Klenow polymerase and ligated into Smal-digested pAK80, thus obtaining pJM334 and pJM335, respectively.

Primer extension. Cells were grown in rich medium to an OD₄₅₀ of 0.6. RNA from E. coli was extracted by the hot method previously described (method II in reference 10). RNA from L. lactis was extracted by the method developed for Bacillus thuringiensis (12). Primer extensions on 1 µg of total RNA were performed as described previously (4).
satisfied by uridine. To clone upp from L. lactis MG1363, a
HindIII genomic library (3), constructed in pUN121 (26), was
used to transform BM604. From selective mineral plates
supplied with uracil, 32 clones were isolated. They were
screened for the presence of the uracil requirement, and 14
clones had the expected phenotype: they were able to grow
with but not without uracil added to the plates. The 18 uracil
prototrophic transformants are most likely pyrF clones. Plas-
mid DNAs from the 14 different uracil-dependent clones were
extracted and retransformed into BM604. They were all able to
confer the uracil-dependent growth phenotype to the host. The
restriction endonuclease maps were constructed, and all plas-
mids contained more than one HindIII insert. Although the
HindIII pattern varied, the plasmids all had a 2.9-kb HindIII
fragment in common. Furthermore, this fragment could be
digested with BglII, and fragments of 2.5 and 0.4 kb could be
identified (not shown). Moreover, plasmids were obtained with
the 2.9-kb HindIII fragment cloned in both orientations with
respect to the vector. Two plasmids, designated pJM300 and
pJM306, with the 2.9-kb HindIII cloned in opposite directions,
were selected for further analysis.

Different internal deletions were constructed in order to
define the location of upp. The gene could be localized on an
internal 1.9-kb NcoI-XbaI fragment on the 2.9-kb HindIII
fragment (Fig. 1).

To confirm that the cloned DNA in pJM300 and pJM306 are
indeed from L. lactis MG1363, Southern blot experiments,
with the two plasmids as probes, were conducted. The 2.9-kb
HindIII fragment can be identified on the chromosomal DNA
from L. lactis MG1363 with either of the two plasmids as
probes (not shown).

E. coli S0106 (wild type with respect to upp), three BM604
derivatives harboring pJM300, pJM306, and pUN121, respec-
tively, and L. lactis MG1363 were analyzed for the presence of
UPRTase activity. For enzyme assays, L. lactis MG1363 was
grown in SA glucose, while E. coli strains were grown in
minimal medium supplied with uridine. The UPRTase activi-
ties were assayed, and the results are presented in Table 2.
UPRTase activity was detected in L. lactis MG1363, and the
specific activity was comparable to that found in an E. coli
wild-type strain. Furthermore, the UPRTase activity was
linked to the upp-complementing plasmid, since the upp
strains harboring pJM300 and pJM306 were shown to have
UPRTase activity. Since the UPRTase activities of strains harboring
either of the upp clones are essential the same, it is reasonable
to believe that the expression of the upp gene in E. coli is
due to the activity of a promoter residing on the lactococcal DNA
and not to transcripts initiated in the vector. In conclusion,

these results demonstrate that the upp gene from L. lactis
subsp. cremoris MG1363 has been cloned and is expressed in E.
coli.

DNA sequence determination. With different deletion deriv-
avatives of pJM300 as DNA templates, and both pUN121
universal primers and specific upp primers, both strands of the
entire upp gene, including the flanking regions, were se-
quenced. The resulting sequence, including the putative
UPRTase amino acid sequence deduced from the DNA, is
presented in Fig. 2. The amino acid sequence was aligned with
the known sequence of UPRTase of E. coli (4), and the
similarity was found to be 56% (not shown). From the amino
acid sequences of 22 different enzymes facilitating reactions
involving PRPP, a consensus sequence for PRPP binding sites
has been proposed (4, 16). This consensus sequence has been
aligned with the putative UPRTase amino acid sequence from
L. lactis MG1363. The potential PRPP binding site is under-
-scored in Fig. 2. Both upstream and downstream of the upp
gene, open reading frames can be detected. Computer
searches were performed in the Swiss Prot release 24.0 data-
base, using the Genetics Computer Group software package,
version 7.0 (8), for protein sequences showing homology to the
putative amino acid sequences of the two open reading frames
upstream and downstream of the upp gene. The deduced
amino acid sequence upstream of upp showed high homology
to the Na<sup>+</H</sup><sup>+</sup> antiporter of Enterococcus hirae (39). The
similarity between the 379 amino acid residues of the lacto-
coccal open reading frame and the 383 amino acids residues
deduced from the sequence of nah from E. hirae was found to
be 48%. Moreover, both potential proteins have 59% hydropho-
bic amino acid residues, and the numbers and positions of
potential membrane-spanning domains are identical. The 150-
amino-acid sequence of the open reading frame downstream
from upp showed 23% identity to a 173-codon potential open
reading frame (orfA) localized on the IS150 element in E. coli

![FIG. 1. Physical map of pJM300. The upp-containing HindIII fragment is shown together with the vector pUN121 (26). Selected restriction endonuclease sites are shown. The three open reading frames deduced from the sequence are presented. The scale is in base pairs. The site of transcription initiation of the upp gene in L. lactis is indicated by an arrow.](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>UPRTase activity(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1363</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>S0106</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MB112 (upp)</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>BM604</td>
<td>pUN121</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>BM604</td>
<td>pJM300</td>
<td>83</td>
</tr>
<tr>
<td>BM604</td>
<td>pJM306</td>
<td>79</td>
</tr>
</tbody>
</table>

\(^a\) Determined in crude extract from cells grown at 30°C and expressed as nanomoles of UMP formed per minute per milligram of total protein at 30°C.
(28). The best score, however, was a 63-codon potential open reading frame in the end of DNA fragment AL9 from L. lactis MG1363 (28). The DNA sequences were, except for an insertion and deletion of one base pair, identical over a stretch of 190 nucleotides. Whether the homology extends beyond the 190 nucleotides is not possible to decide, since no additional sequence data for AL9 are available. This segment on AL9 is placed upstream and in the opposite direction of a gene encoding an exported protein of unknown function (28). Since

the upp gene is placed at the same position as the gene encoding the exported protein with respect to the putative insertion sequence element, it can be concluded that upp is not closely linked to AL9.

A potential ribosome binding site complementary to the 3' end of L. lactis 16S rRNA (22, 38) was found 8 nucleotides upstream of the putative AUG start codon for the UPRTase. This pattern is consistent with the findings for other lactococcal genes (28). The nucleotides complementary to the 16S rRNA and the AUG start codon are underscored in Fig. 2. To determine whether upp is expressed monoscanntronically by a promoter in front of the open reading frame, various DNA fragments containing DNA sequences upstream of upp were cloned in front of a promoterless lacLM gene from Leuconostoc mesenteroides on plasmid pAK80. This transcriptional fusion vector is a shuttle vector maintained by the citrate plasmid replicon in L. lactis (27) and the replicon from pACYC184 in E. coli. Furthermore, it confers erythromycin resistance to the host (19). MG1363 was transformed with pAK80, pJM333, pJM334, and pJM335, and the amounts of β-galactosidase expressed from the four strains were determined (Fig. 3). Only strains harboring pJM333 and pJM334 gave rise to β-galactosidase activity. These results demonstrate that the upp gene is expressed from a promoter positioned downstream of the NarI site and upstream of (or overlapping) the NarI site (Fig. 2 and 3).

To map the transcription initiation site, primer extension experiments were conducted with RNA extracted from an E. coli strain harboring pJM370 and an L. lactis strain harboring PG*371. Interestingly, the E. coli and L. lactis RNA polymerases seem to recognize two different sites in front of the upp open reading frame. In E. coli, transcription is initiated at position 168 (shown by an asterisk Fig. 2). This position is preceded by two domains showing homology to the −35 and −10 regions of the σ70 promoter of E. coli (14), starting at positions 134 and 159, respectively (overscored in Fig. 2). The two sequences are separated by 19 bp. In MG1363, transcription of the upp gene is initiated at position 87. This finding is in accordance with the conclusions based on the results obtained with the promoter fusions. Interestingly, only a −10 sequence showing significant homology to the σ', like promoters in lactococci (38) can be established in connection with the initiation at position 87. Spaced by 17 bp, a sequence with very poor homology to known −35 sequences might be postulated. At position 881, 23 bp downstream of the stop codon of the putative UPRTase open reading frame, the start of a potential

FIG. 2. Nucleotide sequence of the upp gene and the deduced amino acid sequence of UPRTase. The promoters mapped by primer extension are indicated; the first nucleotide to be transcribed in E. coli is marked by an asterisk, whereas the transcriptional initiation site in L. lactis is marked +1. The −35 and −10 regions of the putative promoters are underscored, as are the inverted repeats of the potential terminator. The putative translational start signals and the amino acid sequence showing homology to PRFP binding domains are underscored. The sequences recognized by NarI and PvuII are indicated.

FIG. 3. Physical map of the upp promoter region fused to lacLM in pAK80. The fusion point between the upp and lacLM genes is indicated. β-Galactosidase expression from MG1363 harboring the different plasmids is presented at the right. The activity is given as OD420(OD420/minute).
terminator structure can be identified. The free energy forming the stem-loop structure was determined (33) and estimated to 
\(-22.6\) kcal (\(-94.6\) kJ/mol). It should be mentioned that the 
stem is unusually long compared with known terminators from 
E. coli (7).

Construction of an upp deletion on the chromosome of L. lactis. The gene inactivation system for gram-positive bacteria developed by Biswas et al. (5) was used to construct an upp deletion. The broad-host-range plasmid pG*host4 contains a temperature-sensitive origin of replication, resulting in inability to replicate at 37°C (23). pG*host4 transformants of 
MG1363 were selected on erythromycin plates at 28°C. The 
restrictive temperature was chosen as 35°C, since MG1363 is 
unable to grow at 37°C. At 35°C, the cells were unable to grow 
in the presence of erythromycin, whereas the cells grew 
normally in the absence of the drug. This finding encouraged 
us to use pG*host4 as a vector to deliver upp deletions or 
mutations to the chromosome of L. lactis MG1363 by homol-
ogous recombination. To obtain a deletion of upp on the 
chromosome, we constructed pG*378. This plasmid carries the 
NcoI-XbaI fragment as shown in Fig. 1 but is devoid of the 
main part of the upp coding region because of an EcoRI-
EcoRV deletion. After transformation of MG1363, integrants 
were selected at 35°C. Integration was expected to be homol-
ogous single crossover either up- or downstream of the upp 
open reading frame. By lowering the temperature, the excision 
of the plasmid was promoted, thereby producing a double- 
crossover event. By keeping the erythromycin selection pres-
sure at 28°C, the excised plasmids were forced to be main-
tained in the cells. Depending on the locations of the two 
recombination events, two different situations could arise. 
Either pG*378 is re-created, leaving the wild-type sequence on 
the chromosome, or the wild-type sequence is excised, leaving 
the upp deletion on the chromosome. By analyzing the plas-
mids present after the double-crossover event, it is possible to 
delete a duplication mutant without screening by Southern blot 
analysis or phenotypic changes. The plasmids of six individ-
ually obtained clones were extracted and mapped by restriction 
endonuclease 
digestion. On the basis of the maps, three 
plasmids were shown to have rescued the wild-type system, 
indicating that the upp mutation has been established on the 
chromosome. The plasmids from two of these strains were 
cured by growth in the absence of erythromycin at high 
temperature. The presence of the upp deletion on the chro-
mosome was verified by Southern blotting, and the two plas-
mid-cured strains were shown to be identical with respect to 
the upp deletion (not shown). The upp strain was designated 
MB112. To obtain a pyrimidine-requiring derivative of MB112, 
pIp55, a plasmid unable to replicate in lactococci carrying an 
internal fragment of pyrF from L. lactis MG1363 (1), was 
transformed into MB112. By selection on M17 plates supplied 
with erythromycin and uridine, the potential pyrF strain was 
selected. The strain was designated MB113. The pyrimidine 
requirement was verified by plating on SA glucose minimal 
medium with erythromycin in the absence and in the presence 
of uridine. The mutant is able to grow only in the presence of 
uridine.

The phenotype of the upp mutation. The effect of the upp 
mutation was analyzed in five ways: (i) assay of UPRTase 
activity in crude extract from MB112 (upp), (ii) uracil excretion 
from MB112 (upp), (iii) the effect of the pyrimidine analog FU 
on growth, (iv) incorporation of labeled uracil into nucleic 
acids, and (v) growth of the pyrimidine auxotrophic mutant 
MB113 (upp pyrF) with different pyrimidine sources.

Table 2 clearly shows that a deletion of the upp gene on the 
chromosome (strain MB112) leads to a total loss of measur-
able UPRTase activity.

It has been reported that an E. coli strain carrying an upp 
mutation excretes uracil to the medium (29). To test whether 
L. lactis carrying an upp mutation excretes uracil to the 
medium, the amount of uracil present in the medium after 
growth of MB112 compared with the wild-type strain MG1363 
was measured by using a bioassay as described in Materials and 
Methods. It was found that MB112 (upp) excretes uracil to a 
concentration of 0.35 ± 0.01 µg/ml in minimal medium, 
whereas the wild-type strain seemed to excrete uracil to a 
concentration of 0.17 ± 0.01 µg/ml when grown overnight.

The pyrimidine analog FU is toxic for most bacteria (25). A 
characteristic of upp mutants in most organisms is their 
resistance to FU, since UPRTase catalyzes the conversion into 
5-fluorouridine monophosphate, the first step in the pathway 
leading to the very toxic 5-fluorodeoxyuridine monophosphate.

L. lactis MG1363 (wild type) and MB112 (upp) were streaked 
on minimal plates supplied with FU in various concentrations 
in order to test their sensitivities to this analog. At a concen-
tration of 0.3 µg/ml, FU was lethal to wild-type cells, whereas 
the upp mutant was resistant. At concentrations above 0.3 
µg/ml, growth of the upp mutant was significantly inhibited and 
only extremely small colonies were formed on FU-containing 
plates. This finding indicates that despite the loss of UPRTase 
activity, FU is still metabolized by MB112.

To test whether uracil is incorporated into nucleic acids even 
in the upp strain, the incorporation rates of [2-14C]uracil in 
strains MG1363 and MB112 were measured. The rates were 
determined to be 130 and 4 pmol of uracil per min, respecti-
vely, at an OD540 of 1. This result confirms the conclusion, 
based on FU sensitivity, that even in the absence of UPRTase 
activity, uracil is still metabolized. The main metabolic pathway 
is, however, the conversion to UMP by the upp gene product.

Strain MB113 (upp pyrF) is a pyrimidine auxotrophic deriv-
ative of MB112 which was constructed in order to analyze the 
ability of uracil and other pyrimidines to support growth. Only 
uridine and cytidine were found to serve as sole pyrimidine 
activates of MB112 which was constructed in order to analyze 
the ability of uracil and other pyrimidines to support growth. 
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catalyzing other reactions involving PRPP. The three UPR Tases are extremely similar in the 16-amino-acid putative PRPP binding domain. They deviate from the general PRPP consensus sequence in positions 1, 4, 5, 9, and 16. The most radical of these changes in the UPRTases are the acidic amino acid found instead of glycine at position 1 and the proline found at position 9. Moreover, this proline is found in all the UPRTases described so far, including the UPRTase of Bacillus subtilis, which recently has been cloned in our laboratory (unpublished data), and S. salivar us (11). In all other PRPP-binding proteins known so far, the residue at that position is aspartic acid.

We have previously shown that uracil, uridine, cytidine, deoxycytidine, and deoxyuridine are able to support growth of a pyrimidine auxotrophic mutant (24). In the present report, we show that UPRTase encoded by upp is required for the utilization of uracil, deoxyuridine, and deoxycytidine as sole pyrimidine sources, whereas uridine and cytidine have alternative pathways (Fig. 4). Furthermore, the existence of an alternative pathway for the metabolism of uracil is demonstrated. The results obtained strongly suggest that in addition to the upp-dependent formation of UMP, uracil may be metabolized through UdR to dUMP (Fig. 4). As expected, such a pathway is not sufficient for supporting growth of a pyrimidine auxotrophic mutant, since deoxyribonucleotides are not oxidized to the corresponding ribonucleotides (25). To our knowledge, this is the first time such a pathway has been demonstrated to function in thymine prototrophic bacteria without a deoxyribose-1-phosphate donor present. This finding can be explained in two ways: the internal concentration of deoxyribose-1-phosphate is high in L. lactis MG1363, or the KM value for deoxyribose-1-phosphate of the thymidine phosphorylase is very low.

As discussed above, UPRTase is responsible for the ability of the cells to utilize exogenous uracil in the growth medium as the sole pyrimidine source. The UPRTase activity is also involved in salvaging uracil, which seems to be formed by internal pyrimidine turnover during bacterial growth, since increased amounts of uracil are excreted to the medium in an UPRTase-deficient strain. In E. coli, an upp mutant was shown to excrete uracil to a concentration of 0.40 μg/ml at an OD450 of 1 (13). In conclusion, these results clearly demonstrate the importance of the upp-encoded UPRTase activity.

Promoter fusions and primer extensions located a promoter immediately in front of the open reading frame encoding UPRTase. The upp gene seems to be transcribed as a single cistronic message, flanked by two unrelated open reading frames. The genomic organization of the lactococcal upp gene thus differs from that in E. coli, in which the upp gene is followed by the uracil transporter gene ura4 (2). Both L. lactis and E. coli RNA polymerases can transcribe the upp gene from L. lactis, but interestingly, transcription initiation takes place at two different sites on the DNA in front of the open reading frame. Both promoters have excellent 10-10 sequences, but whereas the promoter recognized by E. coli RNA polymerase also has an acceptable 35 sequence, no such domain can be identified in the promoter recognized by the lactococcal RNA polymerase (38).

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